

# RAT OSTEOPONTIN

## Immunoperoxidase Assay for Determination of Osteopontin in Rat Samples

### DIRECTIONS FOR USE

Version3 **L25.0-1C1**

For Research Use Only, NOT for Diagnostic Purposes

***Please Read this Package Insert Completely Before Using This Product***

### INTENDED USE

The osteopontin (PONT) test kit is a highly sensitive two-site enzyme linked immunoassay (ELISA) for measuring PONT in biological fluid of rats.

### PRINCIPLE OF THE ASSAY

The principle of the double antibody sandwich ELISA is represented in Figure 1. In this assay the PONT present in samples reacts with the anti-PONT antibodies which have been adsorbed to the surface of polystyrene microtitre wells. After the removal of unbound proteins by washing, the Detection Antibody, biotin conjugated anti-PONT, is added and complexes are formed. Following a wash step, the horseradish peroxidase (HRP) conjugated Streptavidin is added and complexes are formed. After another washing step, the complexes are assayed by the addition of a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB). The quantity of bound enzyme varies directly with the concentration of PONT in the sample tested; thus, the absorbance, at 450 nm, is a measure of the concentration of PONT in the test sample. The quantity of PONT in the test sample can be interpolated from the standard curve constructed from the standards, and corrected for sample dilution.

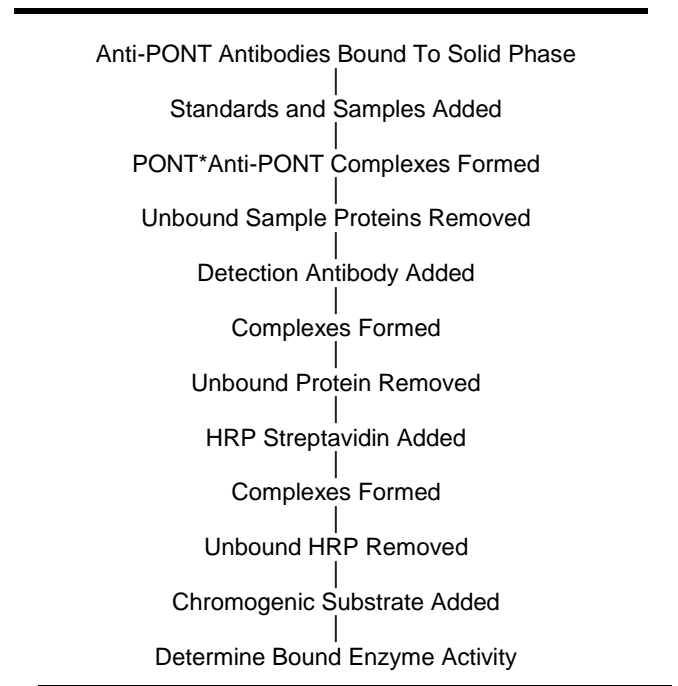


Figure 1.

### REAGENTS (Quantities sufficient for 96 determinations)

#### 1. DILUENT (Running Buffer)

One bottle containing 60 ml of a 1X diluent running buffer.

#### 2. WASH SOLUTION CONCENTRATE

One bottle containing 50 ml of a 20X concentrated wash solution.

#### 3. DETECTION ANTIBODY 100X

One vial containing 150  $\mu$ L of affinity purified anti-Rat Osteopontin antibody conjugated with biotin in a stabilizing buffer.

#### 4. HRP-STREPTAVIDIN 100X

One vial containing 150 µL of HRP conjugated streptavidin in a stabilizing buffer.

#### 5. CHROMOGEN-SUBSTRATE SOLUTION

One vial containing 12 mL of 3,3',5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide in citric acid buffer at pH 3.3.

#### 6. STOP SOLUTION

One vial containing 12 ml 0.3 M sulfuric acid.

**WARNING: Avoid contact with skin.**

#### 7. ANTI-RAT OSTEOPONTIN ELISA MICRO PLATE

Twelve removable eight (8) well micro well strips in well holder frame. Each well is coated with affinity purified anti-Rat PONT.

#### 8. RAT OSTEOPONTIN CALIBRATOR

One vial containing a lyophilized Rat Osteopontin calibrator.

**FOR IN VITRO USE ONLY**

### REAGENT PREPARATION

#### 1. DILUENT

Ready to use as supplied.

#### 2. WASH SOLUTION CONCENTRATE

The Wash Solution supplied is a 20X Concentrate and must be diluted 1/20 with distilled or deionized water (1 part buffer concentrate, 19 parts dH<sub>2</sub>O). Crystal formation in the concentrate is not uncommon when storage temperatures are low. Warming of the concentrate to 30-35°C before dilution can dissolve crystals.

#### 3. DETECTION ANTIBODY 100X

Calculate the required amount of working conjugate solution for each microtitre plate test strip by adding 10 µL detection antibody to 990 µL of 1X Diluent for each test strip to be used for testing. Mix uniformly, but gently. Avoid foaming.

#### 4. HRP-STREPTAVIDIN 100X

Calculate the required amount of working conjugate solution for each microtitre plate test strip by adding 10 µL HRP-streptavidin to 990 µL of 1X Diluent for each test strip to be used for testing. Mix uniformly, but gently. Avoid foaming.

#### 5. CHROMOGEN-SUBSTRATE SOLUTION

Ready to use as supplied.

#### 6. STOP SOLUTION

Ready to use as supplied.

#### 7. ANTI-RAT OSTEOPONTIN ELISA MICRO PLATE

Ready to use as supplied. Unseal Microtiter Pouch and remove plate from pouch. Remove all strips and wells that will not be used in the assay and place back in pouch and re-seal along with desiccant.

#### 8. RAT OSTEOPONTIN CALIBRATOR

Add 1.0 ml of distilled or de-ionized water to the Rat Osteopontin Calibrator and mix gently until dissolved. The calibrator is now at a concentration of 20 ng/ml (**the reconstituted calibrator should be aliquoted and frozen if future use is intended**). Rat Osteopontin standards need to be prepared immediately prior to use (see the following chart). Mix well between each step. Avoid foaming.

Standard	ng/ml	Volume added to 1x Diluent	Volume of 1x Diluent
7	4	100µl Rat PONT Calibrator	400 µl
6	2	250 µl standard 7	250 µl
5	1	250 µl standard 6	250 µl
4	0.5	250 µl standard 5	250 µl
3	0.25	250 µl standard 4	250 µl
2	0.125	250 µl standard 3	250 µl
1	0.0625	250 µl standard 2	250 µl
0	0		500 µl

### STORAGE AND STABILITY

The expiration date for the package is stated on the box label.

#### 1. DILUENT

The Diluent is stable until the expiration date and should be stored at 4-8°C.

#### 2. WASH SOLUTION

The 20X Wash Solution Concentrate is stable until the expiration date. The 1X working solution is stable for at least one week from the date of preparation. Both solutions can be stored at room temperature (16-25°C) or at 4-8°C.

#### 3. DETECTION ANTIBODY 100X

Undiluted Biotin conjugated anti-Rat Osteopontin should be stored at 4-8°C and **diluted immediately prior to use**. The working conjugate solution is stable for up to 1 hour when stored in the dark.

#### 4. HRP-STREPTAVIDIN 100X

Undiluted horseradish peroxidase conjugated streptavidin should be stored at 4-8°C and **diluted immediately prior to use**. The working conjugate solution is stable for up to 1 hour when stored in the dark.

#### 5. CHROMOGEN-SUBSTRATE SOLUTION

The Substrate Solution should be stored at 4-8°C and is stable until the expiration date.

#### 6. STOP SOLUTION

The Stop Solution should be stored at 4-8°C and is stable until the expiration date.

#### 7. ANTI-RAT OSTEOPONTIN ELISA MICRO PLATE

Anti-Rat PONT coated wells are stable until the expiration date, and should be stored at 4-8°C in sealed foil pouch with desiccant pack.

#### 8. RAT OSTEOPONTIN CALIBRATOR

**The lyophilized Rat Osteopontin calibrator should be stored at 4C or frozen until reconstituted. The reconstituted calibrator should be aliquoted out and stored frozen (Avoid multiple freeze-thaw cycles).** The working standard solutions should be prepared immediately prior to use and are stable for up to 1 hour.

#### INDICATIONS OF INSTABILITY

If the test is performing correctly, the results observed with the standard solutions should be within 20 % of the expected values.

#### SPECIMEN COLLECTION AND HANDLING

Blood should be collected by venipuncture. The serum should be separated from the cells after clot formation by centrifugation. For plasma samples, blood should be collected into a container with an anticoagulant and then centrifuged. Care should be taken to minimize hemolysis, excessive hemolysis can impact your results. Assay immediately or aliquot and store samples at -20°C. Avoid repeated freeze-thaw cycles.

##### 1. Precautions

For any sample that might contain pathogens, care must be taken to prevent contact with open wounds.

##### 2. Additives and Preservatives

No additives or preservatives are necessary to maintain the integrity of the specimen. Avoid azide contamination.

##### 3. Known interfering substances

Azide and thimerosal at concentrations higher than 0.1% inhibits the enzyme reaction.

#### MATERIAL PROVIDED

See "REAGENTS"

#### MATERIALS REQUIRED BUT NOT PROVIDED

- Precision pipette (2 µL to 200 µL) for making and dispensing dilutions
- Test tubes
- Microtitre washer/aspirator
- Distilled or Deionized H<sub>2</sub>O
- Microtitre Plate reader
- Assorted glassware for the preparation of reagents and buffer solutions
- Timer

#### ASSAY PROTOCOL

##### DILUTION OF SAMPLES

The assay for quantification of PONT in samples requires that each test sample be diluted before use. For a single step determination a dilution at 1/200 is appropriate for most plasma samples. For absolute quantification, samples that yield results outside the range of the standard curve, a lesser or greater dilution might be required. **If unsure of sample level, a serial dilution with one or two representative samples before running the entire plate is highly recommended.**

1. To prepare 1/200 dilution of sample, transfer 3µL of sample to 597µL of 1X diluent. This gives you a 1/200 dilution of your sample. Mix thoroughly.

#### PROCEDURE

1. **Bring all reagents to room temperature before use.**
2. Pipette 100 µL of
  - Standard 0 (0.0 ng/ml) in duplicate
  - Standard 1 (0.0625 ng/ml) in duplicate
  - Standard 2 (0.125 ng/ml) in duplicate
  - Standard 3 (0.25 ng/ml) in duplicate
  - Standard 4 (0.5 ng/ml) in duplicate
  - Standard 5 (1 ng/ml) in duplicate
  - Standard 6 (2 ng/ml) in duplicate
  - Standard 7 (4 ng/ml) in duplicate
3. Pipette 100 µL of sample (in duplicate) into pre designated wells.

4. Incubate the micro titer plate at room temperature for 2.5 hours. Keep plate covered and level during incubation.

5. Following incubation, aspirate the contents of the wells.

6. Completely fill each well with appropriately diluted Wash Solution and aspirate. Repeat three times, for a total of four washes. If washing manually: completely fill wells with wash buffer, invert the plate then pour/shake out the contents in a waste container. Follow this by sharply striking the wells on absorbent paper to remove residual buffer. Repeat 3 times for a total of four washes.

7. Pipette 100  $\mu$ L of appropriately diluted detection antibody to each well. Incubate the micro titer plate at room temperature for twenty ( $20 \pm 2$ ) minutes. Keep plate covered in the dark and level during incubation.

8. Wash and blot the wells as described in Steps 5/6.

9. Pipette 100  $\mu$ L of appropriately diluted HRP-streptavidin to each well. Incubate the micro titer plate at room temperature for twenty ( $20 \pm 2$ ) minutes. Keep plate covered in the dark and level during incubation.

10. Wash and blot the wells as described in Steps 5/6.

9. Pipette 100  $\mu$ L of TMB Substrate Solution into each well.

10. Incubate in the dark at room temperature for precisely ten (10) minutes.

11. After ten minutes, add 100  $\mu$ L of Stop Solution to each well.

12. Determine the absorbance (450 nm) of the contents of each well. Calibrate the plate reader to manufacturer's specifications.

#### **STABILITY OF THE FINAL REACTION MIXTURE**

The absorbance of the final reaction mixture can be measured up to 2 hours after the addition of the Stop Solution. However, good laboratory practice dictates

that the measurement be made as soon as possible.

#### **RESULTS**

1. Subtract the average background value from the test values for each sample.

2. Using the results observed for the standards construct a Standard Curve. The appropriate curve fit is that of a four-parameter logistics curve. A second order polynomial (quadratic) or other curve fits may also be used.

3. Interpolate test sample values from standard curve. Correct for sera dilution factor to arrive at the osteopontin concentration in original samples.

#### **LIMITATION OF THE PROCEDURE**

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the information contained in the package insert instructions and with adherence to good laboratory practice.

2. Factors that might affect the performance of the assay include proper instrument function, cleanliness of glassware, quality of distilled or deionized water, and accuracy of reagent and sample pipettings, washing technique, incubation time or temperature.

3. Do not mix or substitute reagents with those from other lots or sources.

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